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RESEARCH ARTICLE

Current status and distribution of major RNA viruses infecting onion and garlic crops in Punjab, Pakistan

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ABSTRACT

Onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) are significant bulbous vegetables well known for their kitchen and medicinal values across the globe including Pakistan. *Allium* crops are highly vulnerable to many notorious RNA viruses fit in different genera viz., *Allexivirus*, *Potyvirus*, *Tospovirus* and *Carlavirus*. These viruses are known to inflict colossal and havoc yield losses up to 50% in allium crops wherever they grown. The occurrence of allium viruses in Pakistan has already been reported, but in single crop and only a few studies describe their incidence and distribution. The study examined the current scenario and distribution of major RNA viruses infecting *Allium* crops in Punjab, Pakistan. Field surveys were carried out in 14 districts of Punjab province as well as capital territory Islamabad of Pakistan, for two consecutive growing seasons (2019 and 2020). The 3000 leaf samples collected, 1675 samples (Onion 791, Garlic 884) were found positive for RNA viruses by using specie specific DAS-ELISA and further confirmed through reverse transcriptase polymerase chain reaction (RT-PCR). The results revealed the presence of RNA viruses with overall disease incidence of 52.7% and the highest disease incidence was observed as *Onion yellow dwarf virus* (OYDV) 58% following *Irish yellow spot virus* (IYSV) 41.8% respectively in onion crop during 2019-2020. Similarly, in garlic crop overall disease incidence of RNA viruses was noticed as 58.9% and the highest disease incidence of OYDV was observed as 45%, following IYSV 32.4% and GarV-C 22.5% respectively during 2019-2020. Comparatively, disease incidence and geographical distribution of viruses was variable in surveyed regions of both crops during surveyed period, whereas OYDV and IYSV were most prevalent viruses infecting onion and garlic crops and GarV-C found only in garlic. The current study also concluded the importance to disseminate the information/awareness among the farmers regarding viral diseases and their management by using different strategies including use of resistant genotypes.

Keywords: *Allium* crops; RNA viruses; ELISA; RT-PCR; Disease incidence; Virus distribution

INTRODUCTION

Allium genus belongs to family *Alliaceae* is considered as utmost popular and leading monocotyledonous genera, having onion and garlic as patent aromatic bulbous vegetable crops widely grown across the globe including Pakistan. *Allium* crops are rich source of different vitamins, minerals, proteins, carbohydrates and used as spice, flavoring agent in daily diet (Griffiths et al., 2002; Wilson and Adams, 2007; Bouhenni et al., 2021). They have useful properties regarding phyto-pharmaceutical preparations against various human and animal diseases, while antioxidant activity due to presence of polyphenols, flavonoids, tannins, quercetin and organosulphur (Soto et al., 2015; Kim et al., 2018; Omer et al., 2019; Zhao et al., 2021; Karavelioğlu & Hoca, 2022).

In Pakistan onion and garlic is cultivated on an area of 153.8 thousand hectare's and 9.6 thousand hectare's with total production of 2099.6 thousand tonnes and 85,642 tonnes, respectively (GOP, 2020-21). Both crops are economically important for agriculture sector and Pakistan was ranked among top five onions and garlic exporting countries since 2005. Now a days Pakistan has lost this position and importing onion and garlic to meet country's domestic needs from neighboring countries (GOP, 2019-20). Both crops are facing numerous challenges regarding production and vulnerable to many biotic and abiotic stresses. Throught the world onion and garlic crops are subjected to many viral diseases of different genera's i.e., Potyviruses, Tospoviruses, Allexiviruses and Carlaviruses impacting global agriculture sustainability described by King et al., (2012). These RNA viruses cause severe reduction in plant

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stamina by inducing mosaic, pale color stripes, leaf streaks, water soaked lesion, diamond shape eye spots, stunting and dwarfing, which results in reduced yield up to 50% annually (Chodorska et al., 2014; Gilani et al., 2016).

The viruses of genus (*Potyvirus*) *Onion yellow dwarf virus* (OYDV), *Leak yellow stripe virus* (LYSV) and (*Tospovirus*) *Irish yellow spot virus* (IYSV) are considered as main constraint for the successful production of onion and his seed around the globe because of their serious damage in bulb size and quality (Pappu et al., 2009; Bagi et al., 2012; Iftikhar et al., 2013). OYDV cause severe leaf distortion in onion and garlic crops and leads towards heavy yield losses up to 60% (Ward et al., 2009). OYDV has been identified across the globe because of its wide host range including onion, garlic, leek and shallot. LYSV is an aggrassive member of genus *Potyvirus* which induce yellow stripes to mosaic pattern on leaves of garlic, onion and leek, whereas it reduce the yield up to 54% (Parrano et al., 2012). IYSV is an emerging member of tospoviruses which infect allium crops on the global scale and reported wherever the crops grown. IYSV has widespread distribution and produce straw color diamond shape eye spots on onion, garlic, shallot and leek which results severe crop yield losses up to 100% (Gent et al., 2006; Pappu et al., 2009; Turina et al., 2012; Iftikhar et al., 2014; Ahsan and Ashfaq, 2018).

Garlic crop is infected with highly aggarasive members of different genera including (*Tospovirus*) *Irish yellow spot virus* (IYSV), (*Potyvirus*) *Onion yellow dwarf virus* (OYDV), *Leak yellow stripe virus* (LYSV), (*Allexivirus*) *Shallot virus* X (ShV-X), *Garlic virus* X (GarV-X), *Garlic virus* C (GarV-C), *Garlic virus* D (GarV-D), *Garlic virus* B (GarV-B), *Garlic virus* A (GarV-A), *Garlic virus* E (GarV-E), *Garlic mite borne filamentous virus* (GarMbfV), (*Carlavirus*) *Garlic latent virus* (GLV) and *Shallot latent virus* (SLV) have been reported worldwide (Nam et al., 2015; Bereda et al., 2017; Abraham et al., 2019; Dabrowska et al., 2020; Prajapati et al., 2022). Garlic bulb are the only source material for subsequent crops due to its vegetative propagation nature, therefore, chances of accumulation of garlic viral complex increases the spread to generations through bulbs and leads toward reduce bulb weight and quality stated by Cremer et al., (2021). The Potyviruses, Allexiviruses and Carlaviruses cause synergistic affects and form a complex known as garlic viral complex leads to severe yield losses by deterioting bulb weight and quality (Katis et al., 2012; Mansouri et al., 2021a).

In *Allium* crops, accumulation of RNA viruses in bulb used for propagation and polyphagous insect vector transmission has cardinal role in low production (Das et al., 2021; Baudry et al., 2021). Potyviruses and carlaviruses disseminated through aphid (*Myzus persicae*) non-persistently and favorable environmental conditions enhance the transmission rate (Perotto et al., 2014; Jayasinghe et al., 2021), while tospoviruses transmitted in persistent manner through thrips (*Thrips tabaci*) (Jenser & Szénási 2004; Schwartz et al., 2010; Birithia et al., 2013), and allexiviruses transmitted by (*Aceria tulipae*) a mite semi-persistently (Dhall, 2015; Jangra et al., 2021; Mansouri et al., 2021b). Previous reports based on serological detection followed by RT-PCR showed that these viruses occurred endemically causing mixed infection demonstrated by Sevik & Akcura, (2013). RNA viruses infecting *Allium* crops in Pakistan have significant importance because mosaic diseases reduce yield and bulb quality. Previous records regarding incidence of allium viruses in onion and garlic crops in Pakistan provide little information (Gilani et al., 2016; Ahsan & Ashfaq, 2018). Studies on current status, occurrence and distribution of these viruses are demanding for efficient management on the basis of knowledge about pathogen etiology. In Pakistan there is scant knowledge especially regarding RNA viruses' distribution, diversity and their impact on commercial crops. The principal objective of recent study was to examine the current status, disease incidence as well as distribution of major RNA viruses infecting onion and garlic crops.

MATERIAL AND METHODS

Field survey and sample collection

Field surveys were carried out at farmer fields in 14 districts of Punjab province viz; Multan, Vehari, Khanewal, Jhang, Sahiwal, Kasur, Sheikhupura, Gujranwala,Narowal, Sialkot, Rawalpindi, Chakwal, Attock, Jhelum and the capital territory Islamabad, Pakistan during 2019 and 2020 (Fig. 1). Leaf samples were collected on the basis of random stratified design (RSD) described by Khan et al., (2015) to investigate disease occurrence of major allium viruses. A total of 10 samples (symptomatic 05 and 05 asymptomatic) were collected from each site consisting of one leaf petiole

Fig 1. Map showing surveyed districts of onion and garlic crops during 2019 and 2020 in Punjab, Pakistan.

labeled with necessary information followed by zigzag pattern and placed on ice box. A total of 3000 (onion: 1500 and garlic: 1500) fresh leaf samples were collected displaying virus/virus like characteristic symptom (yellow spot, leaf streaking, straw colored spots, diamond shaped eye spots, water soaked chlorotic lesion, mosaic pattern, stunting and dwarfing) describrd by Oliveira et al., (2014) from 300 different field locations. These plant samples were maintained at Plant virology laboratory, Department of Plant Pathology MNSUA, Multan. All collected samples were rinsed by fresh water to remove apparent contamination, dried on silica gel, and preserved at 4 °C for serological assay.

Serological analysis

DAS-ELISA was performed through commercially available virus specific antisera ELISA kits; OYDV (Art. No.160275, Bioreba AG, Switzerland), IYSV (SRA 60500/0500 Agdia Inc., USA) and GarV-C (Cat #: V112-K1 Nano Diagnostics, USA) as described (Dovas et al., 2001; Shahraeen et al., 2008). Each virus specific IgG was diluted at ratio of 1:1000 in coating buffer and 200 µL/well was distributed in microtiter plate. Each ELISA plate was incubated for 4 hours at 30 °C after wrapping by parafilm tape. Careful washing was performed with PBST for 3-4 times to dispose additional IgG. Controls and sap extracts were pocessed as narrated in (3:2:1), while these extracts and controls were carefully divided into microtiter plate in each well @ 200 µL, plate was placed for night at 4 °C. The plates were washed again as mentioned above and diluted conjugate IgG (1:1000) was dispensed into microtiter plate @ 200 µL/ well, whereas incubated at 4 °C for overnight following 3-5 time washing with wash buffer. *p*NPP solution with concentration of 1 mg/mL was distributed in each well following dark incubation for 30 minutes at room temperature. Virus concentration was seen at absorbance value 405 nm using ELISA plate reader (Multiskan # 5118170 Thermo-fisher Scientific, USA). Intensity of color indicates virus concentration and ELISA reading value A_{405} more than mean of negative control were considered as positive described by Ashfaq et al., (2014) and ELISA positive samples were preserved at -20 °C for further test RT-PCR.

Disease incidence and virus distribution

Relative disease incidence of major RNA viruses was founded on ELISA results through following proportionate demonstrated by Asad et al., (2022).

Disease incidence %

$$= \frac{\text{Total no. of ELISA positive samples}}{\text{Total no. of tested samples}} \times 100$$

Some DAS-ELISA resultant samples along with some healthy were further verified with RT-PCR for results authentication.

Statistical analysis

The mean values of disease incidence and geographical distribution of major allium viruses in each district was performed, whereas statndard errors of mean was calculated in Statistix 10 software.

Reverse transcriptase polymerase chain reaction (RT-PCR)

ELISA resultant onion and garlic samples along with some healthy were verified through RT-PCR for OYDV, IYSV and GarV-C confirmation. The samples were ground into powder for RNA extraction using pestle and mortar in liquid nitrogen. Total RNA was extracted as method described by Malinowski (1997) from 100 mg of plant leaf using TRIzol Reagent kit as the manufacturer instruction. The attained RNA was re-suspended in 100 µL nuclease free water and calculated using Nanodrop spectrophotometer (Thermo-Fisher Scientific, USA) at ratio *A*260/*A*280 nm and preserved at -80 °C. Two step RT-PCR was done using cDNA synthesis Kit (Cat#K1612 Thermo-Fisher Scientific, USA) along with virus-specific downstream reverse primer viz: ` OYDVVKBR37-5`-GTCTCYGTAATTCACGC-3 stated by Arya et al., (2006), IYSV-R35-5`- CTCTTAAACACATTTAACAAGCA-3` used by Iftikhar et al., (2013) and AllexNABP-R75-5-`CCYTTCAGCATATAGCTTAGC-3` narrated by Chen et al., (2004) (Table 1) according to manufacturer information. Reverse transcription was performed using total RNA 2 µL, 1 µL of reverse primers 1 µL of Revert Aid Reverse Transcriptase enzyme (Thermo-Fisher Scientific, USA), 2 µL RT Buffer, 1 µL dNTP's and RNase free water up to 12.5 µL volume (Ahsan et al., 2021). The reaction consisted of the following step I: reaction of 5 min at 65 °C and cold shock of 60 second, step II: reaction of 1 hour at 42 °C (variable), while final extension was done at 75 °C for 10 minutes. Finally obtained amplicons were used for 1% gel electrophoresis pre-stained with ethidium bromide and bands of desired fragment length were visualized under gel documentation system. Amplified cDNA product was preserved at -80 °C for further use.

RESULTS

Field survey and serological analysis

Fields survey results revealed that onion and garlic plants showing characteristic symptoms i.e., yellow necrotic spots, leaf stripes, leaf streaks, straw colored diamond shaped eye spots, water soaked chlorotic lesion, mosaic pattern, stunting and dwarfing of leaves were infected with RNA viruses (Fig. 2). Vector of these viruses i.e., aphid and thrips

Table 1: List of primers used in RT-PCR to detect major RNA viruses infecting onion and garlic crops in Punjab, Pakistan

Primer Name	Group/Virus	Sequence 5'-3'	Region/Expected size	Annealing Temp.	Reference
OYDVVKBF36	OYDV	ATAGCAGAAACAGCTCTTA	Nib-3'UTR/1.1kb	48°C	Arya et al., (2006)
OYDVVKBR37		GTCTCYGTAATTCACGC			
IYSV-F34	IYSV	TAAAACAAACATTCAAACAA	Nib-3'UTR/1.1kb	55°C	Iftikhar et al., (2013)
IYSV-R35		CTCTTAAACACATTTAACAAGCA			
AllexCP+-74	Allexiviruses	TGGRCXTGCTACCACAAYGG	CP-NABP/750bp	57°C	Chen et al., (2004)
AllexNABPR-75		CCYTTCAGCATATAGCTTAGC			

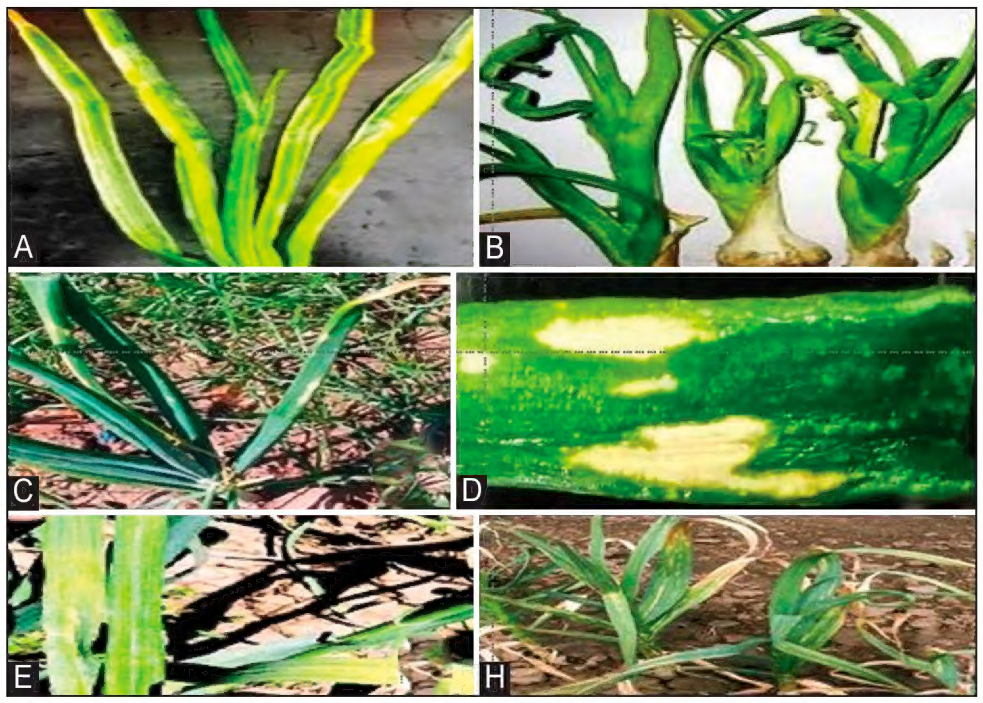

Fig 2. Characteristic symptoms of major RNA viruses observed during field survey of onion and garlic crops in Punjab, Pakistan. A and B (Potyviruses), C and D (Tospoviruses), E and H (Allexiviruses).

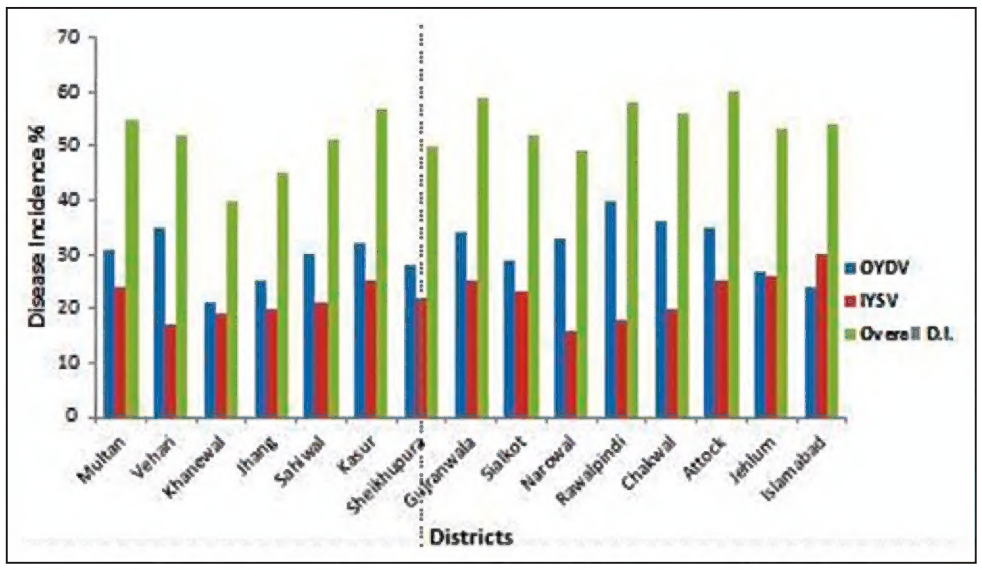

Fig 3. Disease incidence percentage and geographical distribution of major RNA viruses infecting onion crop during 2019-2020 in Punjab, Pakistan.

were also observed during field survey. Based on ELISA results out of 3000 samples 1675 samples (791 from onion and 884 from garlic) were confirmed positive for OYDV and IYSV in onion samples, While OYDV, IYSV and GarV-C from garlic samples during season 2019 and 2020.

Disease incidence and distribution of major RNA viruses infecting onion

Serology results revealed that a total of 791/1500 samples were confirmed positive against OYDV (460) and IYSV (331) from onion crop during 2019-2020 in surveyed region. The overall disease incidence was recorded as 52.7% followed by OYDV (58%) and IYSV (41.8%) respectively (Table 2). In terms of geographical distribution the highest disease occurrence was noted in Attock 60%, following Gujranwala 59%, Rawalpindi 58%, Kasur 57%, Chakwal 56%, Multan 55%, Islamabad 54%, Jhelum 53%, Sialkot 52%, Vehari 52%, Sahiwal 51%, Sheikhupura 50%, Narowal 49%, and Jhang 45%, while lowest in district Khanewal as 40% (Fig. 3). The highest disease incidence of OYDV was estimated as Rawalpindi 40%, Chakwal 36%, Attock 35%, Vehari 35%, Gujranwala 34%, Narowal 33%, Kasur 32%, Multan 31%, Sahiwal 30%, Sialkot 29%, Sheikhupura 28%, Jhelum 27%, Jhang 25% and the capital territory Islamabad 24% repectively. The presence of OYDV was confirmed in all districts and disease incidence varied from higher to lower level, whereas the least disesase incidence was observed in district Khanewal as 21% (Table 2). Subsequently, the disease incidence of IYSV was observed in each surveyed region and varied from higher to lower ranges. The highest disease incidence of IYSV in onion crop was determined in district islamabad 30% followed by Jhelum 26%, Attock 25%, Gujranwala 25%, Kasur 25%, Multan 24%, Sialkot 23%, Sheikhupura 22%, Sahiwal 21%, Jhang 20%, Chakwal 20%, Khanewal 19%, Rawalpindi 18% and Vehari 17% respectively, while least disease incidence was noticed in district Narowal as 16% (Table 2). The variable disease incidence and geographical distribution of OYDV and IYSV in onion crop was owing to insect vector population and unpredicted climatic conditions. The statistical analysis results revealed that mean values of OYDV and IYSVS were significant, whereas it confirm variations in incidence and geographical distribution as higher to lower level (Table 4).

Disease incidence and distribution of major RNA viruses infecting garlic

During 2019-2020 a total of 884/1500 samples were reacted positive against OYDV (398) followed by IYSV (287) and GarV-C (199) from garlic crops in surveyed region. The overall disease incidence was recorded as 58.9% by following OYDV 45%, IYSV 32.4 and GarV-C 22.5% respectively (Table 3). The highest disease was occurred in district Attock as 66%, following Rawalpindi 65%, Islamabad 63%, Kasur 62%, Multan 62%, Gujranwala 61%, Chakwal 60%, Jhelum 59%, Sialkot 58%, Vehari 57%, Sheikhupura 56%, Sahiwal 55% and Jhang 54%,

Table 2: District wise disease incidence and geographic distribution of major RNA viruses infecting onion crop during 2019 and 2020 in Punjab, Pakistan

Year	Crop Name	Sr.#	Districts visited	GPS locations	Total Tested Samples	Total Infected samples	DAS-ELISA			
							OYDV	GrV-C	IYSV	District D.I %
		1	Multan	30.1575° N-71.5249° E	100	55	31	0	24	55%
		2	Vehari	30.0442°N-72.3441°E	100	52	35	0	17	52%
		3	Khanewal	30.2864°N-71.9320°E	100	40	21	0	19	40%
		4	Jhang	31.2781°N-72.3317°E	100	45	25	0	20	45%
		5	Sahiwal	30.6682°N-73.1114°E	100	51	30	0	21	51%
		6	Kasur	31.1179°N- 74.4408°E	100	57	32	0	25	57%
		7	Sheikhupura	31.7167°N-73.9850°E	100	50	28	0	22	50%
2019-2020	Onion	8	Gujranwala	32.1877°N-74.1945°E	100	59	34	0	25	59%
		9	Sialkot	32.4945°N-74.5229°E	100	52	29	0	23	52%
		10	Narowal	32.1014°N-74.8800°E	100	49	33	0	16	49%
		11	Rawalpindi	33.5651°N- 73.0169°E	100	58	40	0	18	58%
		12	Chakwal	32.9328°N-72.8630°E	100	56	36	0	20	56%
		13	Attock	33.7660°N-72.3609°E	100	60	35	0	25	60%
		14	Jhelum	32.9425°N-73.7257°E	100	53	27	0	26	53%
		15	Islamabad	33.6844°N-73.0479°E	100	54	24	0	30	54%
				Total	1500	791	460	0	331	52.7%
				Overall D.I. %	791/1500	52.7%	58%	0	41.8%	

Table 3: District wise disease incidence and geographic distribution of major RNA viruses infecting garlic crop during 2019 and 2020 in Punjab, Pakistan

Year	Crop Name	Sr.#	Districts visited	GPS locations	Total Tested Samples	Total Infected samples	DAS-ELISA			
							OYDV	GrV-C	IYSV	District D.I %
		1	Multan	30.1575° N-71.5249° E	100	62	28	15	19	62%
		2	Vehari	30.0442°N-72.3441°E	100	57	23	14	20	57%
		3	Khanewal	30.2864°N-71.9320°E	100	53	19	16	18	53%
		4	Jhang	31.2781°N-72.3317°E	100	54	24	13	17	54%
		5	Sahiwal	30.6682°N-73.1114°E	100	55	25	12	18	55%
		6	Kasur	31.1179°N- 74.4408°E	100	62	32	09	21	62%
		7	Sheikhupura	31.7167°N-73.9850°E	100	56	27	06	23	56%
		8	Gujranwala	32.1877°N-74.1945°E	100	61	30	10	21	61%
2019-2020	Garlic	9	Sialkot	32.4945°N-74.5229°E	100	58	26	20	12	58%
		10	Narowal	32.1014°N-74.8800°E	100	53	30	07	16	53%
		11	Rawalpindi	33.5651°N- 73.0169°E	100	65	29	11	25	65%
		12	Chakwal	32.9328°N-72.8630°E	100	60	24	16	20	60%
		13	Attock	33.7660°N-72.3609°E	100	66	23	17	26	66%
		14	Jhelum	32.9425°N-73.7257°E	100	59	27	15	17	59%
		15	Islamabad	33.6844°N-73.0479°E	100	63	31	18	14	63%
				Total	1500	884	398	199	287	58.9%
				Overall D.I. %	884/1500	58.9%	45%	22.5%	32.4%	

while least in district Khanewal and Narowal as 53% respectively (Fig. 4). In terms of geographical distribution disease incidence of OYDV in garlic was observed as 32% in Kasur district followed by the capital territory Islamabad 31%, Narowal 30%, Gujranwala 30%, Rawalpindi 29%, Multan 28%, Sheikhupura 27%, Jhelum 27%, Sialkot 26%, Sahiwal 25%, Chakwal 24%, Jhang 24%, Attock 23%, and Vehari 23% respectively, whereas least occurrence was observed as 19% in district Khanewal (Table 3). The IYSV distribution was observed in all districts and the highest disease incidence was notid in district Attock as 26% following Rawalpindi 25%, Sheikhupura 23%, Gujranwala 21%, Kasur 21%, Chakwal 20%, Vehari 20%, Multan 19%, Sahiwal 18%, Khanewal 18%, Jhelum 17%, Jhang 17%, Narowal 16% and the capital territory Islamabad 14% respectively, while lowest in district Sialkot 12 % (Table 3). The highest disease incidence of GarV-C was estimated in district Sialkot as 20% follwed by Islamabad 18%, Attock 17% Chakwal 16%, Khanewal 16%, Jhelum 15%, Multan 15%, Vehari 14%, Jhang 13 %, Sahiwal 12%, Rawalpindi 11%, Gujranwal 10%, Kasur 9%, and Narowal 7% respectively, whereas, least in district Sheikhupura 6%

Table 4: Mean incidence and distribution of major RNA viruses infecting onion and garlic crops during 2019-2020 in Punjab, Pakistan

Onion	OYDV	IYSV	Overall	Garlic	OYDV	IYSV	Overall	GarV-C
OYDV	1	0	0	OYDV	1	0	0	0
p value	0	0	0	p value	0	0	0	0
IYSV	-0.3079	1	0	IYSV	-0.0631	1	0	0
Mean	0.2642	0	0	Mean	0.8233	0	0	0
Overall	0.7315	0.4234	1	Overall	0.3886	0.4738	1	0
Mean	0.0019	0.1158	0	Mean	0.1523	0.0744	0	0
				GarV-C	-0.4179	-0.3897	0.2545	1
				Mean	0.1212	0.151	0.36	0

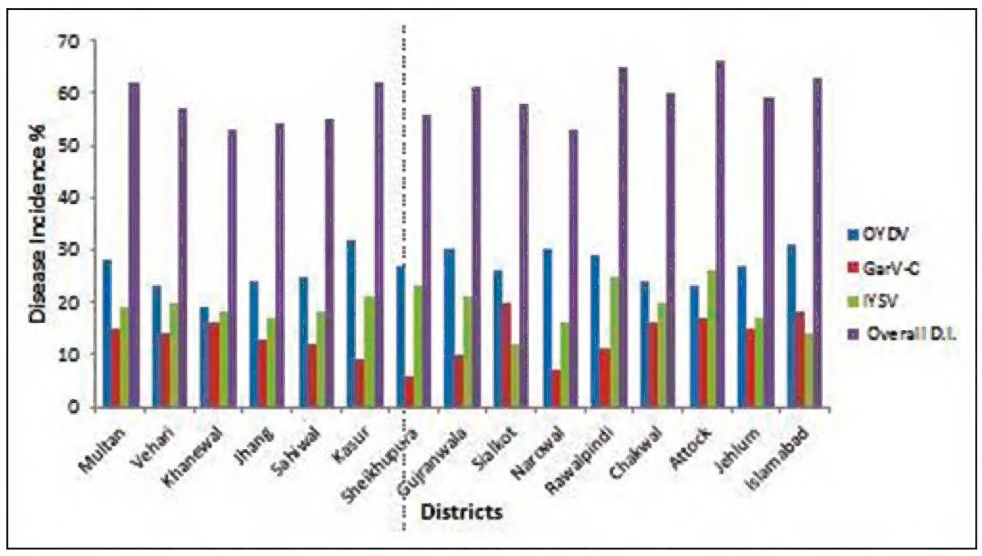

Fig 4. Disease incidence percentage and geographical distribution of major RNA viruses infecting garlic crop during 2019-2020 in Punjab, Pakistan.

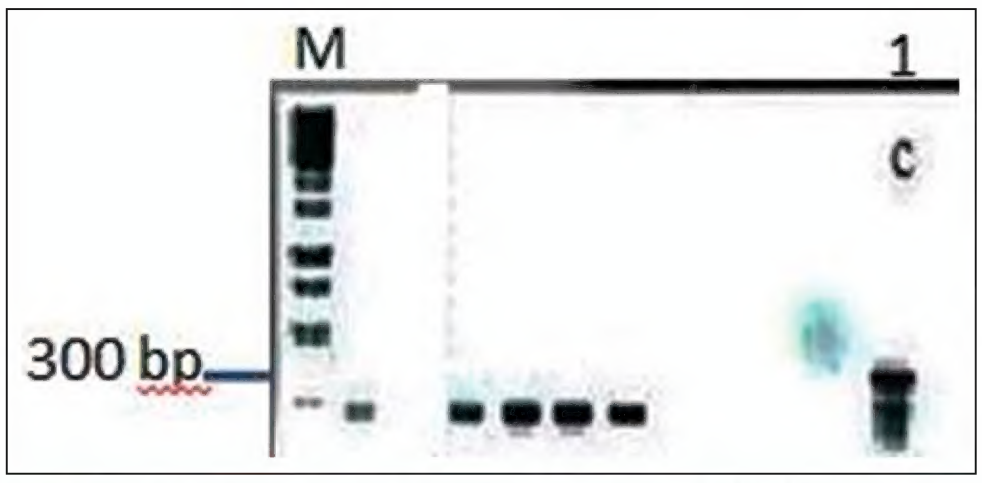

Fig 5. Agarose gel bands showing primer optimization of OYDV infecting onion and garlic crops in Punjab, Pakistan.

(Table 3). Disease incidence varied from area to area in surveyed region and comparatively none of the district was found free of virus infection. Two viruses OYDV and IYSV were most prevalent and widely distributed in onion and garlic crops, while GarV-C was the only in garlic crop during surveyed period. The mean values of OYDV, IYSV and GarV-C occurrence and distribution showed the variation as maximum to minimum that conifrm the significant results (Table 4).

RT-PCR detection

DAS-ELISA results were confirmed through RT-PCR and primer pair OYDVVKBR37-5`-GTCTCYGTAATTCACGC-3, IYSV-R35-5`-CTCTTAAACACATTTAACAAGCA-3` and AllexNABP-R75-5-`CCYTTCAGCATATAGCTTAGC-3` were optimized on the basis of their Nib and CP-NABP gene (Table 1). RT-PCR results evidenced the dominance of OYDV, IYSV and GarV-C and used primer pairs amplified the bands of projected size against OYDV 300 bp (Fig. 3), and IYSV 400 bp (Fig. 4) and (GarV-C 500 bp (Fig. 5), whereas obtained amplicons were stored at -20 °C for further use.

DISCUSSION

Plant viruses are known as major constraints for successful production of cultivated crops and research studies mainly focused on viruses of major crops, while scarce information are available about *Allium* viruses in Pakistan. Onion and garlic are affected with more than 20 notorious viruses of different genera including Allexivirus, Potyvirus, Tospovirus and Carlavirus are detected worldwide (Verma et al., 2015; Mansouri et al., 2021a). Symptoms produced by these viruses are identical to various other diseases and abiotic stresses, so difficult to identify based on symptoms only. Therefore, serological, and molecular methods were deployed in current study for proper detection of targeted viruses.

The observations in current study depicted that OYDV, IYSV and GarV-C viruses has been associated with both economically important crops (onion and garlic). Virus infection rate in current study was (1675/3000) 55.83% although all collected samples exhibit virus/virus like symptoms but resulted rate of detection was low. The results revealed that the highest disease incidence of OYDV in onion was ranged from 25.7% to 30% followed by IYSV 17.9% to 20.6%, whereas highest incidence of OYDV in garlic was ranged from 14.4% to 14.6% followed by IYSV 17.1% to 18% and GarV-C 20.1% to 21% during 2019 and 2020. In 2019-20, overall highest disease incidence 27.6% was recorded for OYDV followed by IYSV with 19.3% in onion crop; while in garlic crop, highest disease incidence was observed 20.5%, 17.6% and 14.5% for GarV-C, IYSV and OYDV, respectively. Gupta et al., (2017) described that samples having no virus detection might strengthen the idea that observed symptoms were due to other virus's

infection. The dominant virus found in current study was OYDV, and these findings are in consistence with previous reports described by Gilani et al., (2016) that occurrence of OYDV and LYSV ranged up to 80-90% in KPK and some districts of Punjab, Pakistan. In current study OYDV and IYSV found to be extensively associated with garlic, whereas infection rate in onion is comparatively low. Garlic crop is deliberated as key host of OYDV and reported with heavy yield losses in various countries across the globe, while current study findings are in confirmation with previous reports (Gawande et al., 2013; Nam et al., 2015; Snihur et al., 2019).

OYDV has wide host range including onion, garlic, leek and shallot make this virus as very destructive pathogen of fresh onion bulb that leads toward huge quantitative and qualitative losses, while high source of infection in onion seed production. OYDV have also found synergistic affects with other viruses of genera allexiviruses and carlaviruses known as garlic viral complex are detected in garlic (Winiarczyk et al., 2014; Khan et al., 2016; Tuzlali et al., 2021). More than 50 aphid species can transmit this virus which is one of the main strength for OYDV wide host range but *Myzus persicae* is the most active vectors (El-Wahab, 2009). Present study results based on disease incidence showed that IYSV presence in field was quite significant and if remained unchecked might cause some serious threats to allium production in Pakistan. IYSV is one of the emerging destructive pathogen of onion, garlic, shallot and leek crops with disease incidence ranges 26-78% which leads serious yield loses up to 80%. IYSV hampered the successful seed production of Allium crops and reported in various countries across the world (Pappu et al., 2008; Ward et al., 2009; Hafez et al., 2012; Bag et al., 2015; Lawande et al., 2016; Manglli et al., 2020).

Disease reports of IYSV have already been described by Iftikhar et al., (2013) in few districts of Punjab, with disease incidence up to 30% in onion. Whereas occurrence of IYSV in garlic was later narrated by Ahsan & Ashfaq (2018) in Pothowar region of Pakistan that evidenced its widespread distribution and findings were in confirmation with present study results. The wide host range of IYSV including cultivated onion, garlic, shallot and leek as well as wild allium species and some ornamentals have been identified (Bulajić et al., 2009; Cramer et al., 2011; Bag et al., 2015; Kamal et al., 2021). The IYSV outbreak in Pakistan is linked with large population of *Thrips tabaci* which transmit virus in persistent and propagative manners, while widespread distribution of thrips in cultivated plants and wide host range trigger the outbreak of pathogen in certain areas. Smith et al., (2011) describe the information on potential host and virus vector relationship within natural vegetation would

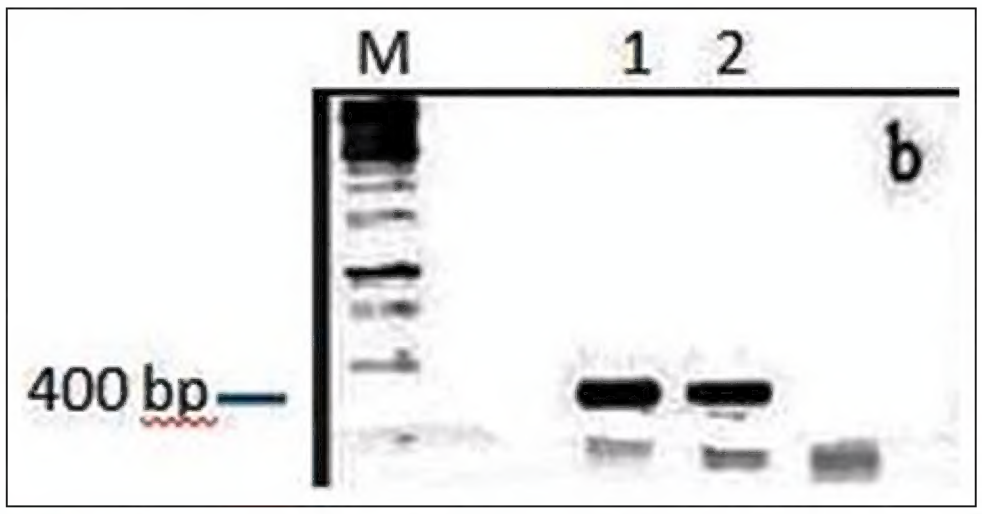

Fig 6. Agarose gel bands showing primer optimization of IYSV infecting onion and garlic crops in Punjab, Pakistan.

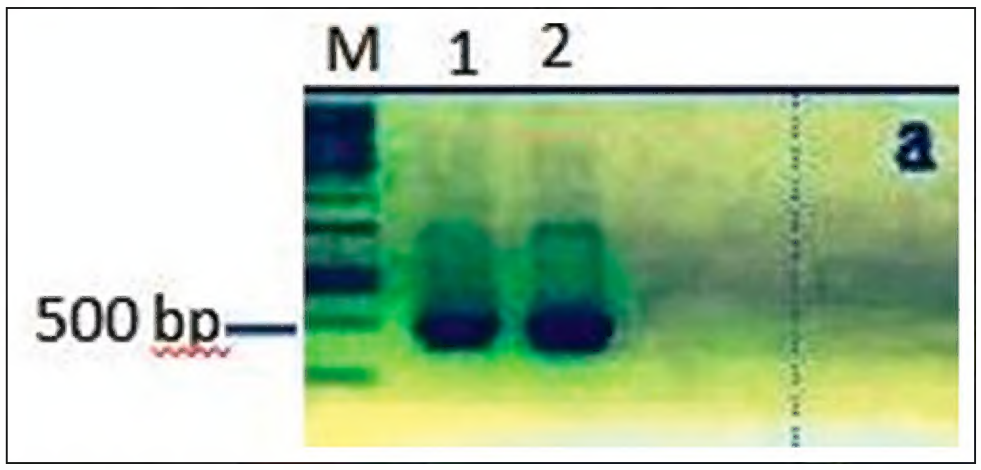

Fig 7. Agarose gel bands showing primer optimization of GarV-C infecting garlic in Punjab, Pakistan.

be mandatory to know the primary source of infection described by Leach et al., (2019).

Mite transmitted *Allexivirus* species GarV-C and GarV-D occurred frequently as multiple infections in garlic, shallot and leek. Allexiviruses disease incidence in the field ranges up to 90% and reported as one of the most occurring viruses of all three genera of garlic viral complex, while detected across the globe (Chodorska et al., 2012; Jemal et al., 2015; Bereda et al., 2017; Abraham et al., 2019; Dąbrowska et al., 2020; Mansouri et al., 2021a; Ayed et al., 2022). Allexiviruses induce mild mosaic, yellow leave strips and leaf deformation usually occurred in mix infection with potyviruses and carlaviruses, whereas allexiviruses Garlic virus B, C, D, and E were found to be most abundant and widely distributed viruses of garlic. The occurrence and infection rate in garlic reach up to 100% because of unhealthy seed and similar observation were made in different countries of the world (Chodorska et al., 2012; Bereda et al., 2016; Katerina et al., 2017; Cremer et al., 2021), while no data was currently available in Pakistan. Present study revealed the detection of GarV-C infecting garlic with occurrence up to 20% in different regions of Punjab, Pakistan. The uneven distribution of allexiviruses linked with bulb storage environment which either favors virus transmission or not narrated by Mann & Minges (1958). GarV-C mostly disseminates during bulb storage conditions through mite (*Acceria tulipae*) and hardly

in the field conditions (Kang et al., 2007; Wijayasekara et al., 2019; Mansouri et al., 2021a). ELISA has been initially considered as sensitive and economical tool for the diagnosis of OYDV, IYSV and GarV-C in onion and garlic (Krauthausen et al., 2012; Santosa & Ertunc, 2020). RT-PCR has been known as more sensitive and efficient method for virus detection (Park et al., 2005; Pappu et al., 2008; Putri & Hidayat, 2020; Tuzlali et al., 2021) and in this study we use couple of tools to detect OYDV, IYSV and GarV-C with specie specific primer pairs for the extension of their Nib, CP, and NABP genes. The amplicons of desired fragment length were obtained from only ELISA positive samples.

The study also discovered that the import of onion and garlic seed/propagating material from neighboring countries such as China (Chen et al., 2004), India (Gawande et al., 2015) and Iran (Shahraeen et al., 2008) affect the production in Pakistan because of frequent viral infection reports in these countries (Baghalian et al., 2010; Sevik, 2018; Prajapati et al., 2022). Furthermore, numerous countries used certified seed program just focused on OYDV elimination from the seed, that's why propagation material trade may or may not be completely virus free and might hold some other viruses. Therefore, chances of reinfection increase several times resulting in significant decrease in yield. The current study found the infection of onion and garlic with multiple viruses that's why there is a strong need to draw a map of distribution these viruses for the main growing areas of Punjab, Pakistan. Frequently new surveys based on wide samples range and growing areas should be conducted to determine novel viruses and their prevalence at national level. The study must be accompanied by means of different tactics to estimate the yield losses caused by onion and garlic viruses solely or in viral complex. The wide dispersal and incidence of *Allium* viruses in studied districts emphasize the necessity of onion and garlic seed certification program in Pakistan, while it is crucial for successful production and improved yield of onion and garlic crops. Therefore it is quite possible to develop joint venture of stakeholders in the trade system targeting to investigate the allium viruses and their losses in major growing areas of the country.

CONCLUSION

The present study was the first to spectacle the current status and distribution of major RNA virus's infecting *Allium* crops in Punjab province and capital territory Islamabad, Pakistan. The study concluded that OYDV and IYSV were the major viruses of onion crop, whereas findings also evidenced the occurrence of OYDV, IYSV and GarV-C as major threat to garlic crop. The viruses' distribution was noticed in both crops and varied in all surveyed regions during the study period. This study provides future direction for further research on existing and new/emerging viruses infecting onion and garlic crops at national level. The information generated from this study will lead to increase awareness among farmers, breeders and researchers about these notorious viruses and their vectors, whereas this information will assist in devising efficient management strategies for better production and improved yield of both crops in Pakistan.

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CONFLICT OF INTEREST

The authors declare that there is no potential conflict of interest.

Author contributions

Conceived the idea and experiments design: M.Z. Hamza, M. Ashfaq, Hasan Riaz & Shafqat Saeed, Experiment performed: M. Z. Hamza, Data Analysis: M.Z. Hamza, M. Ashfaq, Hasan Riaz & Shafqat Saeed, Material/tools/analysis contribution: M. Ashfaq, Manuscript write-up: M.Z. Hamza.

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